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Nitric oxide production in the perifornical-lateral hypothalamic area (PF-LHA) and its influences on the modulation of PF-LHA neurons

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Abstract

The perifornical-lateral hypothalamic area (PF-LHA) is a major wake-promoting structure. It predominantly contains neurons that are active during behavioral and cortical activation. PF-LHA stimulation produces arousal and PF-LHA lesions produce somnolence. Nitric oxide (NO) is a gaseous neurotransmitter that has been implicated in the regulation of multiple pathological and physiological processes including the regulation of sleep. NO levels are higher in the cortex and in the basal forebrain (BF) during arousal. In this study we determined whether NO levels increase in the PF-LHA during prolonged arousal and whether increased NO modulates the discharge activity of PF-LHA neurons.

Experiments were conducted during lights-on phase between 8.00 – 20.00h (lights-on at 8.00h). First, we quantified levels of NO metabolites, NO₂⁻ and NO₃⁻ (collectively called NOx⁻) in the microdialysis dialysates collected from the PF-LHA during baseline (undisturbed rats), 6h of sleep deprivation (SD), and recovery after SD. We further determined the effects of a NO donor, NOC-18, on the discharge activity of PF-LHA neurons in urethane-anesthetized rats.

Overall, SD significantly affected NOx⁻ production in the PF-LHA (one way repeated measures ANOVA, F=7.827, p=0.004). The levels of NOx⁻ increased progressively in animals that were subjected to prolonged arousal as compared to the undisturbed predominantly sleeping animals and decreased during the recovery period. Local application of NOC-18 significantly suppressed the discharge of PF-LHA neurons including a majority of stimulus-on neurons or neurons exhibiting activation during EEG desynchronization. The findings of this study suggest that in the PF-LHA, NO production is elevated during prolonged waking and that NO exerts predominantly inhibitory effects on PF-LHA neurons, especially on those neurons that are active during cortical

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activation. These findings are consistent with a hypothesis that NO in the PF-LHA plays a role in sleep regulation by inhibiting its neurons.

Keywords

Perifornical-lateral hypothalamus; Posterior-lateral hypothalamus; Nitric oxide; Orexin; Sleep; Sleep deprivation

INTRODUCTION

The perifornical-lateral hypothalamic area (PF-LHA) has been implicated in the regulation of many physiological functions, including locomotor activity and sleep-wake control (Gerashchenko and Shiromani, 2004, Datta and Maclean, 2007, McCarley, 2007, Szymusiak and McGinty, 2008, Nunez et al., 2009). PF-LHA contains a heterogeneous population of neurons including those expressing glutamate, hypocretin (HCRT), melanin-concentrating hormone (MCH), and GABA (Bittencourt et al., 1992, Abrahamson and Moore, 2001). Amongst these neuronal types, HCRTergic and glutamatergic neurons have been implicated in the regulation of waking, whereas GABAergic and MCH neurons are sleep-active and have been implicated in the regulation of sleep, especially rapid eye movement (REM) sleep (Lee et al., 2005, Milevskiy et al., 2005, Hassani et al., 2009, Hassani et al., 2010). Electrophysiological recordings from the PF-LHA indicate that this area predominantly contains neurons that discharge with behavioral/cortical activation and are quiescent during nonREM sleep, in addition to relatively smaller populations of state-indifferent, REM-active and nonREM-active neurons (Alam et al., 2002, Suntsova et al., 2007). Although recent studies indicate that HCRTergic and other wake-active neurons within PF-LHA are under adenosinergic and GABAergic inhibitory control (Thakkar et al., 2008, Alam et al., 2010, Rai et al., 2010), the role of other neurotransmitters and neuromodulators that could influence the activity of PF-LHA neurons to promote sleep remains poorly understood.

Nitric oxide (NO) is a ubiquitous intercellular signaling molecule that has been implicated in the regulation of multiple pathological and physiological processes including the regulation of sleep (Zhang and Snyder, 1995, Cooper et al., 1996, Obal and Krueger, 2003, Calabrese et al., 2007, Datta and Maclean, 2007, Stenberg, 2007, Reeves et al., 2008). Because of its short half-life and its ability to diffuse rapidly, NO can nearly simultaneously modulate the activity of a large number of cells in a restricted brain area. Both peripheral and central administrations of NO synthase (NOS) inhibitors decrease spontaneous sleep and delta power, whereas NO donors promote sleep (Kapas et al., 1994, Kapas and Krueger, 1996, Monti et al., 2001, Monti and Jantos, 2004). Local injections of NOS inhibitors into the pedunculopontine tegmentum and the dorsal raphe nucleus decrease, whereas, a NO donor increases both nonREM and REM sleep (Datta et al., 1997, Monti et al., 2001). NO levels are higher in the cortex during spontaneous arousal and in the basal forebrain (BF) during sleep deprivation (SD) (Burlet and Cespuglio, 1997, Cespuglio et al., 2004, Kalinchuk et al., 2006a). Recent electrophysiological studies show that locally increased NO concentration predominantly exerts inhibitory effects on the discharge of BF neurons (Kostin et al., 2008, Kostin et al., 2009). Such inhibition is proposed to induce the compensatory recovery sleep after prolonged waking, since blockade of NO synthesis in the BF impairs recovery sleep subsequent to SD (Kalinchuk et al., 2006a, Kalinchuk et al., 2006b). A role of NO in the PF-LHA on sleep regulation is not known.

In this study we tested a hypothesis that activation of PF-LHA neurons during waking contributes to the local production of NO and that a subsequent NO-induced inhibition of PF-LHA neurons contributes to sleep. In the first set of experiments, we investigated the

effect of prolonged waking on NO production in the PF-LHA by measuring levels of NO metabolites, NO_2^- and NO_3^- (collectively NOx^-), in the dialysate samples collected from this area in animals left undisturbed during lights-on phase, during 6h of SD, and during recovery period following SD. In the second set of experiments, we infused a NO donor, NOC-18, into the PF-LHA via a microdialysis probe and investigated the effect of locally increased NO production on the discharge activity of PF-LHA neurons in urethane anesthetized rats. We found that NOx^- levels in the PF-LHA increased progressively during prolonged waking and that local infusion of a NO donor suppressed the discharge of majority of PF-LHA neurons including stimulus-on/desynchronization-active neurons. These findings are consistent with a role of NO in the PF-LHA in sleep-wake regulation.

EXPERIMENTAL PROCEDURES

Experimental subjects were Sprague-Dawley male rats weighing between 300–350 grams at the time of surgery. These rats were maintained on 12:12h light:dark cycle (lights on at 8h), an ambient temperature of $25 \pm 1^\circ\text{C}$, and with food and water available *ad libitum*. All experiments were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the Veterans Affairs Greater Los Angeles Healthcare System's Institutional Animal Research Committee.

Experiment-1: Determine levels of NOx^- in the PF-LHA

A. Surgical implantations—The experimental procedures used in this study have been described earlier in detail (Alam et al., 1999, Alam et al., 2005). In brief, under surgical anesthesia (Ketamine + Xylazine: 80:10 mg/kg; i.p.) and aseptic conditions, electroencephalogram (EEG) and electromyogram (EMG) electrodes were implanted for polygraphic determination of sleep-waking states in 13 rats. Two microdialysis guide cannulae (23 G stainless steel tubing) were stereotactically implanted such that their tips rested 2 mm dorsal to the PF-LHA (AP -3.0 mm; L 1.4 mm; H 8.0 mm)(Paxinos and Watson, 1998) and were blocked with stylets.

B. Recovery and adaptation—After surgery, rats were placed into home cages in a sound attenuated recording chamber (ambient temperature, $25 \pm 1^\circ\text{C}$, lights on, 08.00h – 20.00h) with food and water available at *ad libitum*. Experiments were conducted after at least 10 days of recovery from surgery and acclimatization of rats with the recording environment. Animals were also acclimatized to human presence and handling manipulations for at least 5 days before the experiments to minimize stress due to human contact and/or experimental procedures. At least two days before the experiment, rats were connected to recording cables.

C. Data acquisition and dialysates collection—At least 12h before the experiment, the stylets of the microdialysis guide cannulae were removed and microdialysis probes (CMA/11, membrane length 1mm and diameter 0.24 mm; CMA/Microdialysis) were inserted into the PF-LHA, fixed, and flushed with artificial cerebrospinal fluid (aCSF; composition in mM, 145 NaCl, 2.7 KCl, 1.3 MgSO_4 , 1.2 CaCl_2 , and 2 Na_2HPO_4 ; pH, 7.2) at a flow rate of 1 $\mu\text{l}/\text{min}$. The time taken by the aCSF solution to travel from the reservoir to the tips of the probes were precisely calculated.

The dialysate samples were collected from the PF-LHA at 30 min intervals between 8.00–20.00h. Starting at 8.00h (lights-on), samples were collected during the first 2h from undisturbed animals as a baseline control, followed by 6h of SD, and 2h of recovery. Rats were sleep-deprived using a gentle handling or environmental enriching procedure (Franken et al. 1991), which included introduction of new objects into the cage in order to keep the

animals occupied and replacing them with new ones when animals appeared becoming drowsy. As an additional control, in a limited number of animals ($n=4$), samples were also collected next day from undisturbed animals between 8.00h–20.00h as time of the day control. In order to avoid the impact of changes in the recovery of the probe on NOx^- levels in samples collected on the second day, the data were normalized to the baseline value of each day.

D. Sleep-wake recording—Amplified and filtered EEG and EMG were continuously digitized and stored on hard-disk of host-computer with the use of an integrated computer interface device (Cambridge Electronic Design 1401; supporting software, Spike 2; London) for subsequent sleep-wake analyses. Sleep-wake profiles of animals during the experimental period were scored manually in 4s epochs in terms of waking, nonREM and REM sleep using standard criteria (Alam and Mallick, 1990).

E. Histology—At the end of the experiment, under deep anesthesia (100mg/kg, i.p., pentobarbital) rats were given heparin (500U, i.p.) and perfused transcardially with 30–50ml of 0.1M phosphate buffer (pH 7.2) followed by 300 ml of 4% paraformaldehyde in phosphate buffer containing 15% saturated picric acid solution. The brains were removed and equilibrated in 10%, 20%, and finally 30% sucrose until they sank. Horizontal sections through the PF-LHA were freeze-cut at 30 μm thickness and immunostained using previously described methods (Kumar et al., 2008) for HCRT. Locations of microdialysis probes were histologically confirmed (figure-1).

F. NOx^- measurements—NO is a free radical with a half-life of 3–5s making it difficult to accurately measure its levels *in vivo*. Since NO is the only known endogenous source of NO_2^- and NO_3^- in the brain (collectively called NOx^-), these metabolites have generally been used as indicators of NO production (Kalinchuk et al., 2006a). We used a nitrate/nitrite fluorometric assay kit (Cayman Chemical Company, Ann Arbor, MI, USA), which has been used for measuring NOx^- levels in earlier studies (Kalinchuk et al., 2006a) following the manufacturer's protocol. The Kit is supplied with a manual that described each step of analysis in detail. In brief, the nitrates in samples were first converted into nitrites utilizing nitrate reductase and then reacted with 2,3-diaminonaphthalene into a fluorescent product, 1(H)-naphthotriazole. After those reactions, fluorescence of the samples was measured by spectro-fluorometer with excitation and emission wavelengths of 360–365nm and 430nm, respectively. The NOx^- levels in dialysates were finally calculated by comparing fluorescence levels of each sample and fluorescence of standards containing known concentrations of NOx^- .

G. Statistical Analysis—The levels of NOx^- at different time intervals during 6h of SD were compared with baseline levels before SD and with that observed during recovery period using one-way repeated measures ANOVA followed by Student-Newman-Keuls test.

Experiment 2: Determine effects of NO on the discharge activity of PF-LHA neurons

A. Surgical implantation—The details of the surgical and recording procedures have been described earlier (Kostin et al., 2008). Briefly, eleven rats were anesthetized by urethane (i.p., 1.5g/kg) and placed in a stereotaxic apparatus. A microelectrode-microdialysis assembly consisting of 2 microwire bundles, each consisting of four 20 μm microwires glued together as a tetrode, and a microdialysis guide cannula was stereotactically implanted into the PF-LHA for simultaneous extracellular recording of its cells and adjacent drug delivery via the microdialysis probe. Screw electrodes were implanted on skull for epidural recording of EEG.

B. Data acquisition—The experiment was started at least 2h after implantation of microelectrode-microdialysis assembly into the PF-LHA. The placement of the microdialysis probe was such that it was adjacent to the microwire bundles and the extracellular environment of the recorded neurons was within the estimated diffusion field of the microdialysis probe (Alam et al., 2005, Kumar et al., 2007). The microdialysis probe was fixed and microwire bundles were advanced adjacent to the side of the exposed microdialysis membrane to minimize the tissue trauma and to ensure maximum stability of the unit recording. The microwire bundles were advanced in ~15µm steps and allowed to stabilize for 15–20min. Each wire was then scanned for the presence of isolated single units (signal to noise ratio >2.0).

First, EEG and the discharge activity of isolated neurons were simultaneously recorded for 30min during aCSF infusion as a baseline. After baseline recordings, either 0.5mM or 1.0mM of a NO donor, NOC-18 (Sigma-Aldrich, USA) was perfused for 30min. After delivery of NOC-18, the perfusion solution was switched back to aCSF and recordings continued for another 30–60 min. EEG and raw action potential signals from microwires were continuously digitized and displayed on a computer monitor using CED 1401/Spike 2 and stored on a disc for subsequent analyses. We found that while both 0.5mM and 1.0mM of NOC-18 produced significant effects on the discharge, 1.0mM NOC-18 produced stronger effects. Therefore, 1mM of NOC-18 was used to determine the effects of NO on most of the PF-LHA neurons, and such neurons were further examined for their responsiveness to tactile stimulation.

C. Tactile stimulation—After examining the effects of NO on the discharge activity of PF-LHA neurons, each neuron was examined for its response to tactile stimulation (figure-4). This procedure involved tail pinching with a consistent pressure using a blunt forceps for 10 – 15s at at-least 100s intervals. Each neuron was subjected to three trials. While pinches were accompanied with EEG desynchronization, during pinching animals did not demonstrate any sign of pain or movement, i.e., were under stable anesthetization.

D. Histology—At the end of experiments rats were sacrificed as described above and brains were extracted. The location of the microdialysis probe and the microwire tracts were histologically confirmed by slicing the brain through the microwire-microdialysis probe tracts. The distribution of the recorded neurons along with their response profiles to tactile stimulation was mapped (figure-5).

E. Data Analysis—Individual action potentials were isolated from the background as well as from other action potentials, if multiple spikes were present, based upon various spike amplitude and shape parameters using spike-2 software. The baseline mean discharge rate/sec of each neuron was calculated from the entire baseline recording. We observed consistent and strongest effects of NOC-18 during the 20–30min of its perfusion. Therefore, we compared the discharge during the last 10 min of the 30min of NOC-18 perfusion with that observed during baseline under similar EEG conditions using paired t-test or Wilcoxon signed rank test, in cases where the data obtained failed normality test.

For evaluation of the effect of tactile stimulation on the discharge activity of neurons, the mean discharge rate during 30 sec preceding stimulus was compared with the mean discharge rate during 10 sec of pinch application (figure-4). Responses were identified as activating or inhibiting if the mean discharge rate increased or decreased, respectively, by more than 20% during pinching vs. pre-pinches period. Neurons were classified as “stimulus-on” and “stimulus-off” if they exhibited >20% increased and decreased discharge, respectively, in response to 2 of 3 trials of tail pinching. Based on spike shape parameters used in an earlier study in urethane anesthetized rats (Mileykovskiy et al., 2005), namely,

long-duration spikes with a long-lasting later positive deflection that was $>0.83\text{ms}$, stimulus-on neurons were further classified into subgroups of putative HCRTergic and non-HCRTergic neurons. Neurons exhibiting a change of $<20\%$ or responding less than 2/3 to tail-pinching trials, were classified as “non-responsive or stimulus-indifferent”.

RESULTS

1. NOx^- levels in the PF-LHA during baseline and SD

The hypothalamic sites where the dialysates for the assay of NOx^- were collected from are shown in figure-1. The probes were mostly localized in the perifornical, dorso-lateral and dorso-medial hypothalamic areas, i.e., in the HCRT neuronal field and a few of them were localized in the ventrolateral and ventromedial hypothalamic areas. Those cases where probes were localized within the HCRTergic field ($n=9$) were grouped together, whereas those cases in which probes were localized only partially or were out of the HCRTergic field ($n=4$, 2 bilateral and 2 unilateral) were grouped together for assessing NOx^- levels in HCRTergic field vs. adjoining areas.

The changes in NOx^- concentrations (mean $\pm$ SEM) in dialysates collected from the PF-LHA during 08.00–20.00h in animals that were left undisturbed or were sleep deprived for 6h are shown in figures 2 and 3. Within the 2h baseline after lights-on, when animals spent most time asleep (waking: first h, $28 \pm 5\%$ and second h, $13 \pm 2\%$), NOx^- concentration during the first h was significantly higher than the level observed during the second h ($1.23 \pm 0.32\mu\text{M}$ vs. $0.65 \pm 0.20\mu\text{M}$, $p<0.05$). The NOx^- concentration during second h was not significantly different from NOx^- levels during 3–10h in undisturbed animals that spent most time asleep as well as during the first 2h of SD in sleep deprived animals. Therefore, NOx^- concentration observed in undisturbed animals during the second h after lights-on was used as baseline for comparison.

There was an overall effect of SD on NOx^- concentration (one way repeated measures ANOVA, $F=7.827$, $p=0.004$). NOx^- level was significantly higher during SD as compared to its levels during baseline as well as recovery period (figure-2). The concentration of NOx^- during recovery period was not significantly different from its baseline level. NOx^- levels in undisturbed animals ($n=4$) during timeframes corresponding to baseline, SD, and recovery period were comparable. In order to determine the temporal relation of NOx^- production to SD, NOx^- concentrations during baseline, 2h blocks of SD (0–2h, 2–4h, and 4–6h), and recovery period were compared. The concentration of NOx^- increased progressively during SD with levels that were significantly higher after 2–4 and 4–6h of SD as compared to baseline and 0–2h of SD. NOx^- concentration during recovery period returned to the baseline level. NOx^- levels in samples collected from probes located outside of the HCRTergic field ($n=4$, 2 bilateral and 2 unilateral sites) during baseline, SD and recovery period demonstrated profiles similar to that observed in samples collected from the HCRTergic field, i.e., a tendency towards elevated levels during SD, although this increase was marginal (figure-2).

2. Effects of NOC-18 on the discharge activity of the PF-LHA neurons

In order to evaluate whether PF-LHA neurons are subject to nitrgergic influences, we determined the effects of 0.5mM and 1.0mM of NOC-18 on the discharge activity of the PF-LHA neurons recorded adjacent to the microdialysis probe used for its delivery. The distribution of recorded neurons along with their stimulus-response profiles is shown in figure-5. Most of the recorded neurons were localized in the perifornical area, dorso-lateral and dorso-medial hypothalamic area, i.e., in areas where HCRT neurons are also localized (de Lecea et al., 1998, Peyron et al., 1998).

The effects of NOC-18 on the discharge activity of individual PF-LHA neurons and on the mean discharge of PF-LHA neurons as a group are shown in figures 6 and 7 respectively. Both doses of NOC-18 (0.5mM; n=31 neurons; 1mM, n=101 neurons) suppressed the discharge activity of PF-LHA neurons. However, as compared to 0.5mM, 1mM NOC-18 produced relatively stronger suppression (20% vs. 11% of their discharge rate) and also affected larger population of neurons (54% vs. 42%), and therefore, was used for studying the effects of NOC-18 on neurons. The neurons examined with 1mM NOC-18 were also characterized for their discharge profiles during tactile stimulation and included, 55 stimulus-on neurons (54.5%), 12 stimulus-off neurons (11.9%), and 34 stimulus-indifferent neurons (33.7%). The response profiles of each neuronal group to NOC-18 is described below:

A. Stimulus-on neurons—Majority of the recorded neurons belonged to this category. NOC-18 significantly suppressed the discharge of stimulus-on neurons as a group (figure-8). While the response of individual neurons varied, majority of neurons (56%) exhibited suppressed discharge, while only a minority of neurons exhibited elevated discharge in response to NOC-18. Of 55 neurons studied, based on spike shape characteristics, 12 neurons were identified as putative HCRTergic neurons (Mileykovskiy et al., 2005). Of these neurons, nine (75%) exhibited suppression, two exhibited activation and one was non-responsive to NOC-18.

B. Stimulus-off neurons—These neurons as a group did not exhibit significant change in response to NOC-18. Overall, these neurons exhibited a more variable response to NOC-18 and a tendency toward suppression in discharge, which was not significant.

C. Stimulus-indifferent neurons—This was the second largest population of neurons recorded. These neurons as a group exhibited significant suppression in discharge in response to NOC-18. However, individually, the response to NOC-18 was variable, with 50% neurons exhibiting suppression and 41% neurons exhibiting no response to NOC-18.

DISCUSSION

The main findings of this study are: a) that NOx^- levels in the PF-LHA are elevated in sleep deprived animals during lights-on phase as compared to conditions when animals are predominantly asleep; and b) that pharmacologically increased local production of NO exerts predominantly inhibitory effects on PF-LHA neurons, particularly the stimulus-on/desynchronization-on neurons in anesthetized preparations. Given that PF-LHA is a wake-promoting area and that NO has been implicated in sleep regulation, we speculate that NO production during sustained waking in the PF-LHA could be a feedback signal, which suppresses local arousal-inducing cells to promote sleep. To confirm this hypothesis, it will be critical to show that NO levels are increased during spontaneous arousal, that NO levels change in response to different ranges of arousal or SD and at different circadian phases, and that manipulations leading to increased or decreased NO production within the PF-LHA affect spontaneous as well as recovery sleep.

Although the levels of NOx^- are an indirect measures of NO, the validity of this approach has been confirmed by directly measuring NO production using diaminofluorescein-2/diacetate staining in the BF (Kalinchuk et al., 2010). This approach has also been used earlier for measuring NO levels in the BF during various behavioral states (Kalinchuk et al., 2006a). This study indicates that increased production of NO during SD is not specific to the BF region. It may be related to the activation of compact groups of wake-active neurons in wake-promoting regions such as BF and PF-LHA, although there might be some site-specific characteristics of NO producing mechanisms. Furthermore, while the animals were

predominantly asleep during the first h after lights-on, NOx⁻ level in the PF-LHA was significantly higher as compared to those observed during the second h or other segments of the lights-on phase with comparable sleep amounts. Since NOx⁻ is more stable than NO, it is plausible that increased NOx⁻ levels at light-onset reflects the accumulation of the increased NO production during prior dark period, when the animals were predominantly awake. We hypothesize that this elevated NO levels may contribute to increased sleep propensity during early phase of lights-on period.

In this study, we did not ascertain which cell type, neuronal or glial, and if neuronal, which neuronal phenotype, and which enzyme, nNOS, eNOS, and/or iNOS, contributed to NO production during SD. A recent study in the BF, however, indicates that NO during SD is produced by neurons, which is mediated by iNOS (Kalinchuk et al., 2010). We found earlier that drug perfused via the microdialysis probe induced Fos-IR in about 500–750µm wide area (Alam et al., 2005, Kumar et al., 2007). As NOx⁻ molecules are relatively small and not strongly bound by known membrane pumps, we might assume that the sampled region has a diameter of 500–750µm. Since the probes were mostly located within the HCRTerigic field, it is reasonable to suggest that activation of HCRT neurons at least partly contributed to NO production during SD. However, PF-LHA contains several other neuronal groups including glutamatergic neurons (Bittencourt et al., 1992, Abrahamson and Moore, 2001). PF-LHA glutamatergic neurons are predominantly wake-active and glutamate has been implicated in the activation of HCRT neurons as well as NO production (Li et al., 2002, Torrealba et al., 2003, Gautier-Sauvigne et al., 2005, Kumar et al., 2005). The activation of PF-LHA glutamatergic neurons during SD may be another source of increased NO production in this area. A limited number of samples were also collected from areas adjacent to the HCRTerigic field with a potential overlap of the diffusion fields of the probes. Therefore, the capability of neurons in areas adjacent to the HCRTerigic field to produce NO cannot be ruled out.

The PF-LHA is critically involved in the regulation of behavioral arousal (see introduction) and contains neurons that are active during behavioral/cortical activation and are quiescent during sleep. In this study, local application of a NO donor significantly suppressed the discharge of PF-LHA neurons. In the BF, where NO levels also increase during SD and contributes to recovery sleep, a NO donor also inhibited its neurons (Kalinchuk et al., 2006a, Kostin et al., 2008). These findings are consistent with a possible sleep-promoting function of NO in wake-promoting regions. A similar role NO has been reported in other brain regions including pedunculopontine tegmentum and dorsal raphe nucleus (Datta et al., 1997, Monti et al., 2001, Kalinchuk et al., 2006a).

In this study, use of an anesthetized preparation provided the advantages of a stable extracellular unit recording and strict comparisons of the discharge activity of recorded neurons during baseline vs. NO donor treatments within a stabilized and homogenous state. Although, the behavior of the cells across spontaneous sleep-wake cycle could not be ascertained, it is likely that the neuronal subgroups identified, i.e., stimulus-on, stimulus-off, and stimulus-indifferent correspond to wake/wake-REM-active, sleep-active, and state-indifferent neurons, respectively, in intact animals. A predominance of stimulus-on or desynchronization-on neurons as reported in this study is consistent with a wake-promoting role of the PF-LHA (see introduction).

While the neurotransmitter phenotypes of the recorded stimulus-on neurons could not be ascertained, based on juxtacellular labeling and discharge characteristics of HCRT neurons in urethane anesthetized rats (Mileykovskiy et al., 2005), it is likely that a subset of recorded neurons were HCRTerigic. NO inhibited 9 of 12 (75%) such neurons. The PF-LHA also contains neurons that have been implicated in the promotion of sleep, especially REM sleep

(Hassani et al., 2009, Hassani et al., 2010). In this study NO produced minimal inhibitory effects on a limited number of recorded stimulus-off neurons, a finding which is consistent with studies showing that sleep-promoting neurons including MCH neurons in the PF-LHA are under weaker inhibitory control than wake-active neurons including HCRT neurons (Alam et al., 2005, Suntsova et al., 2007).

In this study, although NO donor significantly suppressed the discharge of PF-LHA neurons as a group, the responses of individual neurons were variable. This could be due to one or combination of factors: a) that the concentration of NO produced adjacent to the recorded neurons varied; and b) that the state-dependent discharge of PF-LHA neurons is modulated by multiple neurochemical inputs (Kukkonen et al., 2002, Li et al., 2002, Ohno and Sakurai, 2008) and NO-mediated inhibitory tone is one of several factors contributing to the quiescence of PF-LHA neurons during sleep. For example, recent evidence suggests that both GABAergic and adenosinergic inhibitions contribute to the suppression of PF-LHA neurons during sleep (Alam et al., 2010, Rai et al., 2010). NO has also been implicated in the modulation of both GABA and adenosine-mediated responses (Rosenberg et al., 2000, Ferraro and Sardo, 2004). Recent studies show that in the BF, NO promotes the production of adenosine and the inhibitory actions of NO on BF stimulus-on neurons is mediated via an A1 receptor-dependent adenosinergic mechanism (Kostin et al., 2008). Whether NO in the PF-LHA directly contributes to the quiescence of its neurons during sleep or indirectly via adenosinergic or GABAergic mechanisms remains unknown.

In conclusion, this study demonstrates that nitrergic system in the PF-LHA is activated during SD or sustained waking and that elevated NO in the PF-LHA suppresses the discharge activity of its neurons, including stimulus-on/desynchronization-on neurons. These findings are consistent with a hypothesis that NO in the PF-LHA plays a role in the regulation of sleep by inhibiting the activity of its neurons, especially neurons that are active during cortical and behavioral arousal.

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ABBREVIATIONS

aCSF	Artificial cerebrospinal fluid
BF	Basal forebrain
Fos-IR	c-fos protein immunoreactivity
GABA	Gamma-aminobutyric acid
HCRT	Hypocretin also called orexin
MCH	Melanin-concentrating hormone
NO	Nitric oxide
NOS	Nitric oxide synthase
e	endothelial
i	inducible
n	neuronal
NOx⁻	Collective nomenclature for NO metabolites, NO ₂ ⁻ and NO ₃ ⁻

NOC-18	3,3-bis(aminoethyl)-1-hydroxy-1-oxo-1-triazene
NonREM	Non-rapid eye movement sleep
PF-LHA	Perifornical-lateral hypothalamic area
SD	Sleep deprivation

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Figure 1.

(A) Photomicrograph of a representative horizontal section from an animal (40x magnification) showing bilateral locations of the microdialysis probes (large arrows). Brown cell bodies, indicated by small arrows, are HCRT neurons. In this case the probes were localized bilaterally within the HCRT neuronal field. (B) Diagrammatic representation of coronal sections through the PF-LHA showing anatomical locations of the microdialysis probes. Black bars represent probes that were considered within HCRTergic field, whereas gray bars are cases where probes were not considered strictly within the HCRTergic field, although their diffusion fields potentially overlapped partially. DMH, dorso-medial hypothalamic area; LH, lateral hypothalamic area; mt, mamillothalamic tract; 3V, third ventricle.

Figure 2.

(A) Bar diagrams showing percentage changes (mean ± SEM) in NO_x⁻ levels during 6h of SD and recovery period (2h) in sleep-deprived animals and at comparable time intervals in undisturbed animals as compared to their respective baseline levels taken as 100%. The SEM during baseline was calculated by determining percent individual variations in NO_x⁻ levels as compared to the mean baseline value. (B) Bar diagrams showing percent waking during baseline, SD, and recovery in sleep-deprived groups vs. in undisturbed group at comparable intervals. Sleep deprivation significantly increased NO_x⁻ levels, especially in the PF-LHA. *, \$ p < 0.05; **, \$\$ p < 0.01 levels of significance (One way repeated measures ANOVA followed by Student-Newman-Keuls test). *, as compared to baseline; \$, as compared to recovery.

Figure 3.

(A) Bar diagrams showing NOx⁻ concentrations (mean ± SEM) during baseline (BL), different durations of SD and recovery period (REC). NOx⁻ concentration increased progressively depending upon the duration of SD. *, $p < 0.05$, **, $p < 0.01$ levels of significance (One way repeated measures ANOVA followed by Student-Newman-Keuls test).

Figure 4.

Traces of continuous EEG and unit recording showing responses of a typical stimulus-on (unit-1) and a stimulus-indifferent neuron (unit-2) recorded simultaneously (A) and a stimulus-off neuron (B) to tail pinches. Arrows mark the beginnings of tail pinches. Each neuron was subjected to 3 trials. Tail pinching was generally accompanied with transient EEG desynchronization indicating that stimulus-on and stimulus-off neurons were desynchronization-on and desynchronization-off, respectively.

Figure 5.

(A) Photomicrograph of a coronal slice through the PF-LHA showing locations of the microdialysis probe and microwire bundles adjacent to the probe. The recorded neurons were in ~500 μ m range and within the diffusion field of the microdialysis probe. (B) Diagrammatic representation of coronal sections through the PF-LHA showing anatomical locations and phenotypes of the recorded 101 neurons that were studied with 1mM NOC-18. Most of the recorded neurons were localized in the HCRT neuronal field. Black squares, stimulus-on; Blank squares, stimulus-off; and gray squares, stimulus-indifferent neurons; f, fornix; mt, mammillothalamic tract; opt, optic tract; 3V, third ventricle.

Figure 6.

A 120min continuous recording showing a typical microdialysis-unit recording experiment in anesthetized rats with raw discharge as well as discharge rates of two isolated stimulus-on neurons recorded simultaneously during baseline (aCSF perfusion), NOC-18 perfusion, and washout period (aCSF perfusion). Thick and thin lines indicate arrival of NOC-18 at the microdialysis probe and beginning of its washout, respectively. NOC-18 was perfused for 30min. In this example both of the recorded neurons exhibited suppression in discharge during NOC-18 perfusion, although unit-1 exhibited stronger response. The overdrawn waveforms of 20 individual action potentials captured during each condition confirms that same cells were recorded across the experiment and that the suppression in discharge was not due to any change in spike shape parameters or loss of cell.

Figure 7.

(A) Bar diagrams showing the effects of 0.5mM and 1.0mM of NOC-18 on mean ($\pm$ SEM) discharge of the recorded PF-LHA neurons as a group. (B) Percentages of PF-LHA neurons that exhibited decreased (black bars), increased (gray), or no response (white) to NOC-18. While 1mM of NOC-18 was relatively more effective, both doses of NOC-18 suppressed the discharge of PF-LHA neurons. *, **, $p < 0.05$ and < 0.01 levels of significance, respectively.

Figure 8.

(A) Bar diagrams showing the effects of 1.0mM of NOC-18 on the mean ($\pm$ SEM) discharge of the various cell types recorded in the PF-LHA. (B) Percentages of PF-LHA neurons that exhibited decreased (black bars), increased (gray), or no response (white) to NOC-18 in each category. *, **, $p < 0.05$ and < 0.01 levels of significance, respectively.